

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
26 June 2003 (26.06.2003)

PCT

(10) International Publication Number
WO 03/052109 A1

(51) International Patent Classification⁷: C12N 15/82 (74) Agent: KREMER, Simon, M.; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

(21) International Application Number: PCT/EP02/14512

(22) International Filing Date:
18 December 2002 (18.12.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
01130319.5 19 December 2001 (19.12.2001) EP

(71) Applicant and

(72) Inventor: DÜRING, Klaus [DE/DE]; Vorgebirgsweg 33,
50226 Frechen (DE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): MAHN, Andreas [DE/DE]; Alte Windmühle 2, 50129 Bergheim (DE). HANTKE, Sabine [DE/DE]; Neusser Strasse 316, 50733 Köln (DE). PETSCHE, Dagmar [DE/DE]; Erlenweg 11a, 50827 Köln (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/052109 A1

(54) Title: METHOD OF INCREASING THE TRANSGENE-CODED BIOMOLECULE CONTENT IN ORGANISMS

(57) Abstract: The invention relates to a method of obtaining an increase in the transgene-coded biomolecule content in organisms. The method is based on a change, which can be carried out by various procedures, of the distribution of ATP and/or ADP in cells of the organism.

Method of Increasing the Transgene-Coded Biomolecule Content in Organisms

The present invention relates to a method of increasing the transgene-coded biomolecule content in organisms, in particular in plants. The method is based on a change in the distribution of ATP and/or ADP in cells of the organism which can be carried out by various procedures,

Both naturally occurring and synthetic proteins, peptides and nucleic acids have highly interesting properties as regards their uses as active substances and materials, but are often only available in very small amounts. Since it is also often not possible to obtain them efficiently under economic conditions and in sufficient amounts in recombinant host systems, e.g. in bacteria, such as *Escherichia coli*, *Bacillus subtilis*, etc., commercial use cannot be realized. In order to be able to obtain more complex proteins and peptides or nucleic acids which are difficult to produce or cannot be produced at all in lower organisms, cells of higher organisms having an inherent complex protein and nucleic acid biosynthesis machinery are increasingly required as host cells. Transgenic animals, plants, mosses, algae, etc. have offered themselves as new recombinant hosts for some years now. Due to the availability of increasing numbers of well characterized biomolecules from molecular research, use of such hosts is gaining in importance for their production.

However, in host organisms the content of transgene-coded biomolecules does not always lie within the desired range. In particular the yields obtained in the host cells for the production of proteins or nucleic acids on an industrial scale are insufficient. On the one hand, this is due to the regulation of gene expression and, on the other hand, also caused by a degradation of the transgenic products by the host organism. In general, an increase in the expression and an increase in the amount of biomolecules stored in the or-

ganism is desirable for increasing the content of transgenic proteins, peptides and nucleic acids. This could raise the efficiency of the production of biomolecules in transgenic organisms and subsequently facilitate their recovery and purification.

In order to obtain a high content of transgene-coded biomolecules, it is necessary to make use of those regulation mechanisms resulting in an increase of expression and to avoid or eliminate those suppressing the production or degrading the products. The use of strong promoters is a general approach for increasing transcription and thus raising the amount of mRNA made. This is usually also accompanied by an increase in the amount of foreign protein formed.

In order to protect once formed mRNA from an increased turnover, which plays a role in gene silencing, all measures preventing detection of RNA as foreign are suited. Such measures are e.g. the prevention of double-stranded RNA formation, the adaptation of the GC content to that of the host cell and the use of repressor proteins for suppressing post-translational gene silencing (De Wilde, Plant Molecular Biology **545** (2000), 347-359). By adapting the codon usage to that of the host cell it is possible to achieve an increase in translation. The transgene-coded biomolecule content can also be raised by lowering the formation of an endogenous storage protein, as accomplished by Goossens et al., (FEBS Letters **456** (1999), 160-164) by means of the antisense technology. Another possibility of raising the foreign protein expression in transgenic organisms is the construction of fusion proteins between the target proteins and e.g. chaperonins or chaperonin binding domains.

However, it has only been possible thus far to raise the content of the desired transgenic molecules in organisms to some degree by these methods. In order to render the production of biomolecules in transgenic organisms more efficient, a mechanism is highly required which can be used as such or also in addition to said methods and in this connection rai-

ses the transgenically encoded biomolecule content significantly.

The present invention is thus based on the technical problem of providing a means by which an increase in the transgenic biomolecule content can be achieved in organisms, in particular in plants.

This technical problem is solved by the subject matters defined in the claims. The present invention comprises a novel mechanism of increasing the transgene-coded biomolecule content in organisms, such as plants, which is based on influencing the energy metabolism of the cells. It has been found surprisingly that a physiological change can be caused by modifying the distribution of ATP or ADP in the cell so as to achieve a significantly higher content of transgenically coded products in cells of the organism.

ATP is the universal energy carrier of all live cells. Energy in the form of ATP is required for almost all anabolic pathways. In heterotrophic plant cells, ATP is mainly synthesized by means of oxidative phosphorylation in the mitochondria from ADP and inorganic phosphate. Under anaerobic conditions, this is done by means of substrate-level phosphorylation in the cytosol. ATP is transported out of the mitochondria via the mitochondrial ADP/ATP transport protein which is one of the best studied membrane proteins. The mitochondrial ADP/ATP transport protein catalyzes exclusively the ATP export in return for the import of ADP.

In the case of heterotrophic vegetable storage tissues a comparatively large amount of ATP is taken up into the storage plastids to energize biosynthesis steps only occurring there, such as starch or fatty acid biosynthesis. This uptake is catalyzed by a plastidiary ATP/ADP transport protein which is localized in the inner coat membrane and enables the ATP uptake in return for the ADP release.

In order to analyze the effect of modified plastidiary

ATP/ADP transporter activities on the carbohydrate balance, transgenic potato plants having increased or reduced transporter activity were produced in the experiments resulting in the present invention.

The amount of endogenous plastidiary ATP/ADP transporter in potatoes (AATP1, *Solanum tuberosum* St) was reduced by means of antisense inhibition. Part of the AATP1,St-coding cDNA was introduced in antisense orientation into the potato genome. This cDNA was controlled by the constitutive CaMV 35S promoter. Various independent lines having in each case individually reduced activity of the plastidiary ATP/ADP transporter being obtained. The activity of the plastidiary ATP/ADP transporter was thus reduced to 64 % to 79 % as compared to that of non-transgenic control plants. The transgenic potato plants showed no phenotypic changes in the region of the aboveground green tissues. In contrast thereto, the morphology of the tubers was markedly altered (branched tubers) and the starch content dropped to about 50 % as compared to the non-transgenic control plants (Tjaden et al., Plant Journal, 16 (1998), 531-540). Summarized, due to the reduced ATP/ADP transporter activity comparatively less ATP was taken up into the plastids and consequently less starch was produced.

Furthermore, transgenic potato plants having an increased activity of the plastidiary ATP/ADP transporter were produced by introducing the cDNA for the plastidiary ATP/ADP transporter from *Arabidopsis thaliana* (AATP1,AT) in sense orientation into the potato genome under the control of the 35S promoter. This led to various independent lines each having individually increased activity of the plastidiary ATP/ADP transporter. The measured activity of the plastidiary ATP/ADP transporter was between 130 and 148 % in the various lines as compared to those in non-transgenic control plants. The transgenic potato plants showed no phenotypic changes in the region of the aboveground green tissues. However, the starch content in the tubers was increased by up to about 150 % as compared to the control (Tjaden et al., supra). Summarized,

due to the increased ATP/ADP transporter activity comparatively more ATP was taken up into the plastids and therefore more starch was produced.

There is reason to suppose that the change in the ATP or ADP concentrations in certain parts of a plant cell has considerable effects on the cell metabolism and the regulation of genes. It was thus investigated in the studies conducted in connection with the present invention whether such a change also influences the protein content in the plant cells. For this purpose, transgenic potato plants of the Desirée variety were produced e.g. by means of the gene constructs described in Tjaden et al. (*supra*) to either reduce via "antisense" or increase via "sense" constructs the ATP/ADP transporter activity. The resulting transgenic plants were partially hyper-transformed using another transgene. The plants obtained were subjected to a proteinchemical analysis, and the content of different foreign proteins and whole protein in tuber extracts was determined. It turned out that there was a marked increase in the foreign protein content in transgenic plants (cf. below Examples 1-2).

Thus, the present invention relates to a method of increasing the transgene-coded biomolecule content in organisms, preferably in plants, which is characterized by changing the distribution of ATP and/or ADP in cells of the organisms (as compared to the original situation).

An increase in the content of transgene-coded biomolecules is understood to mean every increase in the concentration of said biomolecules in an extract obtained from the tissues of the transgenic organisms as compared to the content in an extract obtained from organisms which do not show the change in the distribution of ATP and/or ADP in the cells according to the present invention. For example, this increase can be effected by an enhanced accumulation of the biomolecules in one or more cell compartments, such as the endoplasmic reticulum, the plastids, the vacuoles, the lysosomes, the mito-

chondria, the cell nucleus, the Golgi apparatus, the peroxisomes, the cytosol and others. In a preferred embodiment of the described method, the content of transgene-coded biomolecules is raised selectively, the content of endogenous biomolecules in cells of the organisms being not changed significantly. This increase can be constitutive or regulated temporally, locally or inducibly.

Within the meaning according to the present invention the transgene-coded biomolecules relate to both proteins and peptides as well as to nucleic acid molecules. In particular peptides, proteins and nucleic acids which are not expressed naturally in the respective target organism are mentioned. However, peptides, proteins and nucleic acids which are not expressed naturally in their present form are also transgene-coded biomolecules. They include e.g. all forms of modified or non-native proteins, peptides and nucleic acids such as hybrid proteins, chimeric proteins and chimeric nucleic acid constructs. Fragments of proteins, peptides or nucleic acids also represent transgenic biomolecules within the meaning according to the present invention. In particular proteins, peptides or nucleic acids whose expression pattern was modified in the respective host organism shall also be transgenically encoded biomolecules within the meaning according to the invention. Examples thereof are proteins, peptides and nucleic acids whose expression is not regulated naturally in the present form. For example, the natural expression thereof can be regulated temporally, locally or inducibly in another way. In particular, the change of the expression of the sequences coding for the biomolecules can also show as an increase or reduction of the expression rate. Nucleic acids are in particular all deoxyribonucleic acids and ribonucleic acids. They are preferably available as ribozymes, single-stranded or double-stranded oligonucleotides or also as relatively long-chain nucleic acid molecules. Particularly preferred proteins are antibodies, aptamers, receptors, enzymes, growth factors, hormones and specific antigen molecules for use in diagnosis, therapy and the prevention of diseases, such as viral diseases or cancerous diseases. Such proteins

may be e.g. interferons, immunoglobulins, growth hormones, insulin, collagen, plasminogen activator, blood factors such as factors I to XII, histocompatibility antigens, enzymes, tumor marker proteins and antibodies specific thereto as well as viral antigens and antibodies specific thereto.

The organisms suitable for use in the method according to the invention may contain one or more transgenes and express them in parallel or sequentially. The parallel expression of several transgenes is conceivable via the control of the coding sequences by constitutive and/or inducible promoters. A sequential expression can be achieved by the regulation of the gene expression of several transgenes in an organism, which can be induced in different ways.

The organisms suitable for the method according to the invention are animals, humans and plants. The term "animals" as used herein comprises preferably mammals, e.g. cows, horses, goats, sheeps, pigs, mice, rats and rabbits. The plants may, in principle, be plants of any species, i.e. both monocotyledonous and dicotyledonous plants. The term "plants" as used herein comprises preferably gramineae, chenopodiacea, leguminosae, brassicaceae, solanaceae, fungi, mosses, and algae. Crop plants, e.g. plants such a wheat, barley, rice, corn, sugar beets, sugarcane, rape, mustard, oilseed rape, flax, safflower, peas, beans, lupins, tobacco, lucerne, soya, bananas, ananas, potatoes, sunflowers, melons, sweet potatoes, spelt, alfalfa, paprika, topinambur, tomatoes, durum wheat or rye are particularly preferred.

In a preferred embodiment, the method according to the invention is characterized in that the activity or concentration of a protein involved in the subcellular distribution of ATP and ADP is increased or reduced in the organism. This protein is usually a protein which is naturally available in the corresponding organism, e.g. the mitochondrial ADP/ATP transport protein, the plastidiary ATP/ADP transporter or the plastidiary triose phosphate/phosphate transporter. A particularly preferred embodiment of the method according to the

invention is one in which the expression of a gene which codes for a protein involved in the subcellular distribution of ATP and ADP is increased or reduced. This gene expression can be modified by methods known to a person skilled in the art. For example, this can be effected by the above changes in the protein concentration and those described in the examples using antisense or sense constructs. A change in the protein activity or concentration can basically be effected via both gene expression and a functional inhibition of the protein activity, e.g. by the expression of binding, inhibiting, neutralizing or catalytic antibodies or other specifically binding and blocking proteins or peptides, by ribozymes, single-stranded or double-stranded oligonucleotides, aptamers, lipids, natural receptors, lectins, carbohydrates, etc.

In the method according to the invention the ATP or ADP concentration in cell compartments can also be influenced by introducing a protein (polypeptide) which is not naturally available in the respective organism. In order to obtain the localization of the protein in the desired cell compartment it may be favorable for the protein to have a signal peptide, so that it can be transported into certain cell compartments of a plant cell. The person skilled in the art is familiar with suitable signal peptides and methods of linking the signal peptides with a desired protein. For example, reference is made to the signal peptide of amylase from barley as to the apoplast (Düring et al., Plant Journal 3 (1993), 587-598), to a murine signal peptide, to the combination of murine signal peptide and the KDEL-ER retention signal as regards ER (Artsaenko et al., Molecular Breeding 4 (1998), 313-319), to the targeting signal of a mammal-alpha-2,5-sialyltransferase regarding the Golgi apparatus (Wee et al., Plant Cell IV (1998), 1759-1768), to the vacuolar localizing signal of a vacuolar chitinase from cucumber as regards the vacuoles (Neuhaus et al., Proc. Natl. Acad. Sci. U.S.A. 88 (1991), 10362-10366), to the ferredoxin transit peptide as to the chloroplasts and plastids, and to the transit peptide of tryptophanyl tRNA synthethase from yeast regarding the mito-

chondria (Schmitz and Lonsdale, *Plant Cell* **1** (1998), 783-791). Basically, the protein involved in the subcellular distribution of ATP and ADP can be administered by various methods, e.g. via media, such as the culture media, of a plant or of parts thereof, in particular plant cells. However, as pointed out above already, it is preferred to administer the protein to plants or parts thereof in the form of a nucleic acid coding for it, e.g. DNA or RNA. For this purpose, it is necessary for the nucleic acid to be available in an expression vector or to be ligated with sequences thereof. In this connection, it can be favorable for this vector or these sequences to enable an expression of the nucleic acid in cell compartments. Such expression vectors or sequences are known to the person skilled in the art. For example, reference is made to Svab et al., *Proc. Natl. Acad. Sci. U.S.A.* **87** (1990), 8526-8530; Khan and Maliga, *Nature Biotechnology* **17** (1999), 910-915; and Sidorov et al., *Plant Journal* **19** (1999), 209-216.

Methods of constructing the expression vectors containing the desired gene, e.g. for a plastidiary ATP/ADP transporter from *Arabidopsis thaliana* (AATP1,At) in expressible form are known to the person skilled in the art and also described in common standard works (cf. e.g. Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). The expression vectors can be based on a plasmid, cosmid, virus, bacteriophage or another vector common in genetic engineering. These vectors may have further functional units which effect stabilization of the vector in the plants, for example. If used for plants they may contain left-border and right-border sequences of agrobacterial T-DNA so as to enable stable integration into the genotype of plants. A termination sequence may also be present which serves for the correct termination of transcription and the addition of a poly-A sequence to the transcript. Such elements are described in the literature (cf. Gielen et al., *EMBO J.* **8** (1989), 23-29) and can be exchanged as desired.

The person skilled in the art is familiar with suitable promoters for the expression of the gene coding for the desired protein. The promoters include e.g. the cauliflower mosaic virus 35S promoter (Odell et al., *Nature* **313** (1995), 810-812), the *Agrobacterium tumefaciens* nopaline synthase promoter and the mannopine synthase promoter (Harpster et al., *Molecular and General Genetics* **212** (1988), 182-190).

The increase or decrease of the above-described protein activities can be effected constitutively or temporally, locally or be induced by certain stimuli. A temporally or locally limited or inducible increase or decrease of the protein activities also suppresses the changes in the tuber morphology, described by Tjaden et al. (*supra*).

Thus, another preferred embodiment of the method according to the invention is characterized in that the expression of the gene whose product causes a change in the distribution of ATP and/or ADP in cells of the organism is regulated in the organism temporally, locally or inducibly. For example, the gene coding for the desired protein can be linked with an inducible promoter, which permits e.g. the control of the synthesis of the desired protein, e.g. in a plant, at a desired time. Suitable promoters are known to the person skilled in the art and they comprise e.g. the anaerobically inducible Gap C4 promoter from corn (Bülow et al., *Molecular Plant-Microbe Interactions* **12** (1999), 182-188), PR promoters such as L-phenylalanine ammonium lyase, chalcone synthase and hydroxyproline rich glycoprotein promoters, inducible by ethylene (Ecker and Davies, *Proc. Natl. Acad. Sci. U.S.A.* (1987), 5202-5210) and a dexamethasone-inducible chimeric transcription induction system (Kunkel et al., *Nature Biotechnology* **17** (1990), 916-918), the IncW promoter from corn inducible by saccharose or D-glucose (Chen et al., *Proc. Natl. Acad. Sci. U.S.A.* **96** (1999), 10512-10517). Reference is also made to Dalta et al., *Biotechnology Annual Review* **3** (1997), 269-290, and Gatz and Denk, *Trends in Plant Science* **3** (1998), 352-358. Furthermore, promoters are suited which permit local regulation of the expression, i.e. only in cer-

tain plant parts or organs. Such promoters are e.g. the pata-tin promoter from potato (Liu et al., Molecular and General Genetics 223 (1990), 401-406) (tuber-specific), the napin promoter from allseed rape (Radke et al., Theoretical and Applied Genetics 75 (1988), 685-694) (embryo-specific in the seed), the RolC promoter from *Agrobacterium rhizogenes* (Yokoyama et al., Molecular and General Genetics 244 (1994), 15-22) (phloem-specific), the TA29 promoter from tobacco (Kriete et al., Plant Journal 9 (1996), 809-818) (tapetum-specific), the LeB4 promoter from *Vicia faba* (Bäumlein et al., Molecular and General Genetics 225 (1991), 121-128) (seed-specific) and the rbcS and cab promoters from petunia (Jones et al., Molecular and General Genetics 212 (1988), 536-542) (leaf-specific or limited to photosynthetically active tissues).

In another preferred embodiment of the method according to the invention the expression of the plastidiary ATP/ADP transporter is raised or lowered. In this connection, the expression can be lowered by introducing an antisense construct suppressing the expression of the endogenous gene, and the expression can be raised by introducing a sense construct. The sense construct may be a gene available on an expression vector for the endogenous transporter e.g. under the control of a strong promoter but also a heterologous gene which codes for a transporter from another organism, preferably a closely related organism.

A large number of cloning vectors which contain a replication signal for *E. coli* and a marker gene for the selection of transformed bacterial cells are available for the production of the expression vectors which shall be introduced into plants. Examples of such vectors are pBR322, pUC series, M13mp series, pA-CYC184, etc. The desired sequence may be introduced into the vector at an appropriate restriction site. The resulting vector is used for the transformation of *E. coli* cells. Transformed *E. coli* cells are cultured in a suitable medium, then harvested and lysed. The vector is then recovered. In general, restriction analyses, gel electrophoresis and further biochemical and molecular-biological me-

thods are used as analytical methods for characterizing the vector DNA obtained. The vector DNA can be cleaved after every manipulation and the DNA fragments obtained can be linked with other DNA sequences. Each vector DNA sequence can be cloned into the same or into other vectors.

A number of methods are available for the introduction of the above expression vectors into a plant cell. These methods comprise transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation means, fusion of protoplasts, injection, electroporation of DNA, introduction of DNA using the biolistic method and further possibilities.

The injection and electroporation of DNA in plant cells do generally not make special demands on the vectors used. It is possible to use simple plasmids such as pUC derivatives. However, if whole plants shall be regenerated from cells transformed in this way, a selectable marker should be present. Suitable selectable markers are known to the person skilled in the art and they comprise e.g. the neomycin phosphotransferase II gene from *E. coli* (Beck et al., Gene 19 (1982), 327-336), the sulfonamide resistance gene (EP-369637), and the hygromycin resistance gene (EP-186425). Depending on the method of introducing the desired gene into the plant cell, further DNA sequences may be required. For example, if the Ti or Ri plasmid is used for the transformation of the plant cell, at least the right border, but often the right and left borders, of the Ti and Ri plasmid T-DNA must be connected as a flanking region with the genes to be introduced.

If agrobacteria are used for the transformation, the DNA to be introduced must be cloned into special vectors, i.e. into either an intermediary vector or a binary vector (cf. the below examples). Due to sequences homologous to sequences in the T-DNA, the intermediary vectors can be integrated into the Ti or Ri plasmid of the agrobacteria by homologous recombination. It also contains the vir region necessary for the

T-DNA transfer. Intermediary vectors cannot replicate in agrobacteria. By means of a helper plasmid, the intermediary vector can be transferred to *Agrobacterium tumefaciens*. Binary vectors can replicate in both *E. coli* and *Agrobacterium*. They contain a selection marker gene and a linker or polylinker, which are surrounded by the right and left T-DNA border. They can be transformed directly into the agrobacteria. The agrobacterium serving as a host cell should contain a plasmid which carries a vir region. The vir region is necessary for the transfer of T-DNA into the plant cell. Additional T-DNA may be present. The agrobacterium transformed in this way is used for the transformation of plant cells.

In order to transfer the DNA into the plant cell, plant explants can usefully be cocultured with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Whole plants can then be regenerated again from the infected plant material (e.g. leaf portions, stem segments, roots, but also protoplasts or suspension-cultured plant cells) in a suitable medium which may contain antibiotics or biocides for the selection of transformed cells. The resulting plants can subsequently be studied for the presence of the introduced DNA. Alternative systems for the transformation of monocotyledonous plants are transformation by means of a biolistic approach, electrically or chemically induced DNA uptake into protoplasts, electroporation of partially permeabilized cells, macroinjection of DNA into inflorescences, microinjection of DNA into microspores, oocytes and pro-embryos, DNA uptake by germinating pollen, and DNA uptake into embryos by swelling (for an overview see Potrykus, Biotechnologie 8 (1990), 535-542). While the transformation of dicotyledonous plants is well established via Ti plasmid vector systems using *Agrobacterium tumefaciens*, more recent studies indicate that monocotyledonous plants are also very well accessible to transformation by means of vectors based on *Agrobacterium*.

In a preferred embodiment, the expression vectors used according to the invention contain localization signals for localization in cell compartments, in particular in endoplasmic

reticulum (ER), apoplasts, Golgi apparatus, plastids, peroxisomes, mitochondria and/or vacuoles. Reference is made to the above statements on the signal peptides. The KDEL-ER targeting peptide, the Golgi localization signal of β -1,2-N-acetylglucosamine transferase (Gnt1), the transit peptide from the small subunit of ribulose bisphosphate carboxylase and/or the vacuolar targeting signal SKNPIN are particularly preferred as localization signals.

In principle, the plant portions desired for the expression of the transgene relate to any plant part, in any case to replication material of these plants, e.g. seeds, tubers or bulbs, rootstocks, seedlings, cuttings, etc.

In principle, the present invention also enables an increase in the expression of transgenes in animals and humans. For this purpose, the above protein can be administered as such or in combination with a signal peptide to animals, humans or cells thereof. Such a signal peptide can be e.g. a murine signal peptide, a combination of a murine signal peptide and the KDEL-ER retention signal, or the targeting signal of a mammalian alpha-2,6-sialyltransferase as regards the Golgi apparatus. Furthermore, the protein can be administered in the form of a nucleic acid coding for it, e.g. DNA or RNA, to animals, humans or cells thereof. Administration in the form of a nucleic acid requires that the latter is present in an expression vector or is ligated with sequences thereof. Reference is made to the above general statements on expression vectors and their production. In addition, reference is made to vectors which are suited for the gene therapy in animals and humans. In them, the nucleic acid can be controlled by an inducible or tissue-specific promoter, such as metallothionein I or polyhedrin promoter. Preferred vectors are e.g. viruses, such as retroviruses, adenoviruses, adeno-associated viruses or vaccinia viruses. Examples of retroviruses are MoMuLV, HaMuSV, MUMTV, RSV or GaLV. Furthermore, the nucleic acid coding for the polypeptide can be transported to the target cells in the form of colloidal dispersions. They comprise e.g. liposomes and lipoplexes (Mannino et al., Bio-

techniques 6 (1988), 682).

According to the invention, the above protein is administered to humans and cells. In principle, the animals may belong to any animal species. They are preferably useful and domestic animals, e.g. cattle, horses, sheep, pigs, goats, chickens, turkeys, dogs, cats, etc.

Examples of transgenes whose expression in animals and humans can be raised are in particular peptides, proteins and nucleic acids. The particularly preferred proteins are antibodies, aptamers, receptors, enzymes, growth factors, hormones and specific antigen and antibody molecules for use in diagnosis, therapy and the prevention of both viral diseases and cancerous diseases. Such proteins are e.g. interferons, immunoglobulins, growth hormones, insulin, collagen, plasminogen activator, blood factors such as factors I to XII, histocompatibility antigens, enzymes, tumor marker proteins and antibodies specific thereto as well as viral antigens and antibodies specific thereto. Examples of nucleic acids are single-stranded and double-stranded RNA or DNA, oligonucleotides and ribozymes.

Brief description of the figures:

Figure 1

Figure 1 shows the NptII protein content in ng/ml in the extract of potato tubers. The lines MBP7sATPT contain the sense gene construct for the plastidiary ATP/ADP translocator from *Arabidopsis thaliana* in transgenic potato plants of the Désirée variety. The lines MPB/aATPT contain the antisense gene construct for the plastidiary ATP/ADP translocator from *Arabidopsis thaliana* in transgenic potato plants of the Désirée variety. Désirée: non-transgenic starting variety Désirée as a control. DK1: transgenic control line var. Désirée only containing the Npt II gene under control of the NOS promoter.

Figure 2

Figure 2 shows the DNA sequences of the primers used and an svFv antibody used according to the invention.

The invention is explained by the following examples.

Example 1: Increase in the expression of Npt II in transgenic potato tubers

The gene constructs described in Tjaden et al. (*supra*) for "antisense" decrease ("MPB/aATPT") or "sense" increase ("MBP/sATPT") of the plastidial ATP/ADP transporter activity in potato tubers were each ligated blunt-end into the opened and filled-in singular HindIII restriction site of the binary vector pSR 8-30 (*cf.* Düring et al., *supra*; Porsch et al., Plant Molecular Biology 37 (1998), 581-585). The two transformation vectors pSR8-30/sATPT were obtained. These two expression vectors were used separately for the transformation of *E. coli* SM10. Transformants were mixed with *Agrobacterium* GV 3101 and incubated at 28°C overnight. (Koncz and Schell, Mol. Gen. Genet. 204 (1986); 383-396, Kocz et al., Proc. Natl. Acad. Sci. U.S.A., 84 (1987), 131-135). Selection was made on carbenicillin, the bla gene necessary for this purpose being available in the above expression vectors. Selected clones of *Agrobacterium tumefaciens* were applied onto detached leaves, cut several times at the middle rib, of potato plants cv. Désirée and the leaves were incubated at 20°C in the dark for 2 days. Thereafter, the agrobacteria were washed off and plant growth substances were added to the potato leaves, so that preferably shoots regenerated. Furthermore, non-transformed cells were killed in the potato leaves by the addition of kanamycin to the plant medium. Growing shoots were cut off and were allowed to grow roots in the medium without plant growth substances but with kanamycin. The further cultivation of the potato plants was performed as usual. On the one hand, transgenic lines including the antisense gene construct and, on the other hand, transgenic lines including the sense gene construct were obtained. The

regenerated potato lines were planted in soil and grown in a greenhouse. After the ripening of the potato plants, the tubers were harvested and stored for the protein-chemical studies.

In order to study the antisense effect on the foreign gene expression, the NptII content in tuber extracts of all lines was compared by means of ELISA, since apart from Désirée all transgenic lines express the Npt II gene under the control of the nos promoter. The detection limit in the sandwich ELISA is at 0.5 ng Npt II/ml extract. The results show that the antisense lines MPB/aATPT/05, MPB/aATPT/13 and MPB/aATPT/22 contain Npt II concentrations which are 9 to 10 times as high as the content of the control. The determined Npt II values are about 0.7 ng/ml in the control DK1 and thus at the same order as those of the sense plants.

It showed that a major increase of the Npt II gene expression could be effected in transgenic potato tubers by using the described antisense constructs according to the invention.

Example 2: Increase in the expression of scFv antibodies in transgenic potato tubers

For this test, the plants described in Example 1 were hyper-transformed with a gene construct which codes for an scFv antibody. The binary vector pLH9000Hyg was obtained by removing by means of restriction digest with XbaI and SpeI the kanamycin resistance-mediating expression cassette of the binary vector pLH9000 (L. Hausmann and R. Töpfer, *Vorträge Pflanzenzüchtung* [Lectures on Plant cultivation] 45 (1999) 155-172). In its place, a hygromycin resistance-mediating expression cassette was inserted which had been produced by amplification by PCR with primers

TCT AGA GAT CAT GAG CGG AGA ATT AA
and

ACT AGT AAT TCC CAT CTT GAA AGA AA

from the binary vector BinHygTOp (GenBank Gl:886843) and subsequent restriction digest using XbaI and SpeI. An expression cassette containing the gene for a single-chain (scFv) antibody having the sequence shown in figure 2 under the control of the CAMV 35S promoter was ligated into the opened SalI restriction site of the binary vector pLH9000Hyg. The transformation vector pLH9000Hyg/scFv was obtained.

This expression vector was used for the transformation of *E. coli* SM10. Transformants were mixed with agrobacterium GV 3101 and incubated at 28°C overnight (Koncz (*supra*)). Selection was made on streptomycin, the aadA gene necessary for this purpose being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* were applied onto detached leaves, cut several times at the middle rib, of the potato plants described in Example 1, and the leaves were incubated at 20°C in the dark for 2 days. Thereafter, the agrobacteria were washed off and plant growth substances were added to the potato leaves, so that preferably shoots regenerated. Furthermore, non-transformed cells in the potato leaves were killed by the addition of hygromycin to the plant medium. Growing shoots were cut off and were allowed to root on the medium without plant growth substances but with hygromycin. The potato plants were further cultivated as usual. Transgenic lines including the antisense gene construct and the scFv gene construct, transgenic lines including the sense gene construct and the scFv gene constructs, and transgenic lines which only included the scFv gene construct were obtained. The regenerated potato lines were planted in soil and grown in a greenhouse. After the ripening of the plants, the resulting potato tubers were stored until the protein-chemical study was conducted.

In order to study the antisense effect on the foreign protein content, the content of scFv in extracts of potato tubers of all lines was compared by means of ELISA. The detection limit in the sandwich ELISA is at 500 ng scFv/ml extract. The results show that the antisense lines MPB/aATPT/05/scFv/05, MPB/aATPT/05/scFv/08 and MPB/aATPT/05/scFv/12 contain scFv

concentrations which are 5 to 10 times as high as the content of the sense lines.

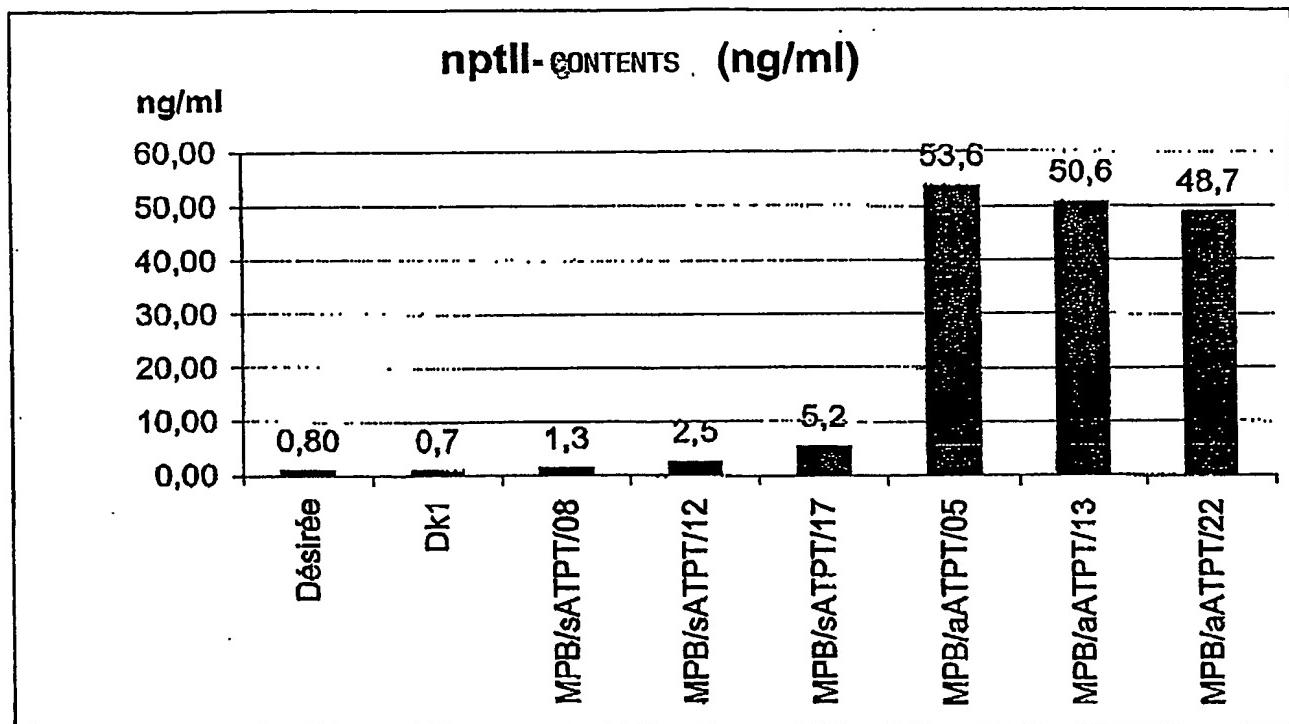
A major increase in the expression of the scFv gene in transgenic potato plants could be effected by using the described antisense constructs according to the invention.

Claims:

1. A method of increasing the content of one or more trans-gene-coded biomolecules in an organism, characterized by changing the distribution of ATP and/or ADP in cells of the organism.
2. The method according to claim 1, wherein the expression of the transgene-coded biomolecules is constitutive or is regulated temporally, locally or inducibly.
3. The method according to claim 1 or 2, wherein several transgene-coded biomolecules are expressed in parallel or sequentially.
4. The method according to any one of claims 1 to 3, wherein the organism is a plant or an animal.
5. The method according to claim 4, wherein the plant comprises gramineae, chenopodiacea, leguminousea, brassicaceae, solanaceae, fungi, mosses, and algae.
6. The method according to claim 4, wherein the plant comprises wheat, barley, rice, corn, sugar beets, sugar cane, rape, mustard, oilseed rape, flax, safflower, peas, beans, lupins, tobacco, lucerne, soya, bananas, ananas, potatoes, sunflowers, melons, sweet potatoes, spelt, alfalfa, paprika, topinambur, tomatoes, durum wheat, rye or batata.
7. The method according to any of claims 1 to 6, wherein the transgene encodes a peptide, protein or a nucleic acid.
8. The method according to any of claims 1 to 7, wherein the transgene codes for antibodies, aptamers, receptors, enzymes, growth factors, hormones, specific antigen and/or antibody molecules, interferons, immunoglobulins, growth hormones, insulin, collagen, plasminogen acti-

vator, blood factors such as factors I to XII, histocompatibility antigens, enzymes, tumor marker proteins and/or viral proteins, ribozymes, single-stranded or double-stranded DNA or RNA.

9. The method according to any of claims 1 to 8, characterized in that the activity or concentration of a protein involved in the subcellular distribution of ATP and/or ADP is increased or reduced in the organism.
10. The method according to any of claims 1 to 9, characterized in that the expression of a gene which codes for a protein involved in the subcellular distribution of ATP and/or ADP is increased or decreased in the organism.
11. The method according to claim 10, characterized in that the expression is constitutive or regulated temporally, locally or inducibly.
12. The method according to claim 10 or 11, characterized in that the expression of the plastidiary ATP/ADP transporter is increased or decreased.

Figur 1:

atggcttcca aacctttct atcttgctt tcactttcct tgcttcctt tacaagcaca	60
tgttta gca gct gat gtg cag ctg gtg gag tct ggg gga ggc tta gtg Ala Ala Asp Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val 1 5 10	108
cag cct gga ggg tcc cg 5 aaa ctc tcc tgt gca gcc tct gga ttc act Gln Pro Gly Gly Ser Arg Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr 15 20 25 30	156
tcc agt agc tt 35 gga atg cac tgg gtt cgt cag gct cca gag aag ggg Phe Ser Ser Phe Gly Met His Trp Val Arg Gln Ala Pro Glu Lys Gly 35 40 45	204
ctg gag tgg gtc gca tat att agt 50 agt ggc agt agt acc atc tac tat Leu Glu Trp Val Ala Tyr Ile Ser Ser Gly Ser Ser Thr Ile Tyr Tyr 50 55 60	252
gca gac aca gtg aag ggc cga ttc acc atc tcc aga gac aat ccc aag Ala Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Pro Lys 65 70 75	300
aac acc ctg ttc ctg caa atg acc agt cta agg tct gag gac acg gcc Asn Thr Leu Phe Leu Gln Met Thr Ser Leu Arg Ser Glu Asp Thr Ala 80 85 90	348
atg tat tac tgc gca aga gat 95 tac ggg gct tat tgg ggc caa ggg acc Met Tyr Tyr Cys Ala Arg Asp Tyr Gly Ala Tyr Trp Gly Gln Gly Thr 95 100 105 110	396
acg gtc acc gtc tcc tca ggt gga ggc ggt tca ggc gqa ggt ggc tct Thr Val Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser 115 120 125	444
ggc ggt ggc gga tcg gac att gag ctc acc cag tct cca gca atc atg Gly Gly Gly Ser Asp Ile Glu Leu Thr Gln Ser Pro Ala Ile Met 130 135 140	492
tct gca tct cca ggg gag aag gtc acc atg acc tgc agt gcc agt tca Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Ser Ala Ser Ser 145 150 155	540
agt gta agg tac atg aac tgg ttc caa cag aag tca ggc acc tcc ccc Ser Val Arg Tyr Met Asn Trp Phe Gln Gln Lys Ser Gly Thr Ser Pro 160 165 170	588
aaa aga tgg att tat gac aca tcc aaa ctg tct tct gga gtc cct gct Lys Arg Trp Ile Tyr Asp Thr Ser Lys Leu Ser Ser Gly Val Pro Ala 175 180 185 190	636
cgc ttc agt ggc agt ggg tct ggg acc tct tac tct ctc aca atc agc Arg Phe Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr Ile Ser 195 200 205	684
agc atg gag gct gaa gat gct gcc act tat tac tgc cag cag tgg agt Ser Met Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln Trp Ser 210 215 220	732
agt aat cca ctc act ttc ggt gct ggg acc aag ctg gag ctg aaa cgg Ser Asn Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg 225 230 235	780
gcg gcc gca gaacaaaaac tcatctcaga agaggatctg aatggatccca Ala Ala Ala 240	829
aagacgaact ctag	843

INTERNATIONAL SEARCH REPORT

Inte al Application No
PCT/EP 02/14512

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 99 58654 A (MAX PLANCK GESELLSCHAFT ;MOEHLMANN TORSTEN (DE); MARTINI NORBERT () 18 November 1999 (1999-11-18) see the whole document ---	1-12
Y	TJADEN J ET AL: "ALTERED PLASTIDIC ATP/ADP-TRANSPORTER ACTIVITY INFLUENCES POTATO (SOLANUM TUBERSUM L.) TUBER MORPHOLOGY, YIELD COMPOSITION OF TUBER STARCH" PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 16, no. 5, 1998, pages 531-540, XP000960526 ISSN: 0960-7412 see the whole document ---	1-12 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the International filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the International filing date but later than the priority date claimed

"T" later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the International search

Date of mailing of the international search report

6 March 2003

20/03/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+31-70) 340-3016

Authorized officer

Grosskopf, R

INTERNATIONAL SEARCH REPORT

Intern'l Application No
PCT/EP 02/14512

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>GAAL TAMAS ET AL: "Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria." SCIENCE (WASHINGTON D C), vol. 278, no. 5346, 19 December 1997 (1997-12-19), pages 2092-2097, XP002233693 ISSN: 0036-8075 see the whole document</p> <p>MAKITA N ET AL: "ATP/ADP switches the higher-order structure of DNA in the presence of spermidine" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 460, no. 2, 29 October 1999 (1999-10-29), pages 333-337, XP004260427 ISSN: 0014-5793 see the whole document</p>	1-12
Y	<p>GEIGENBERGER PETER ET AL: "Tuber physiology and properties of starch from tubers of transgenic potato plants with altered plastidic adenylate transporter activity." PLANT PHYSIOLOGY (ROCKVILLE), vol. 125, no. 4, April 2001 (2001-04), pages 1667-1678, XP002189694 ISSN: 0032-0889</p>	1-12
A	<p>GEIGENBERGER P ET AL: "OVEREXPRESSION OF PYROPHOSPHATASE LEADS TO INCREASED SUCROSE DEGRADATION AND STARCH SYNTHESIS, INCREASED ACTIVITIES OF ENZYMES FOR SUCROSE-STARCH INTERCONVERSIONS, AND INCREASED LEVELS OF NUCLEOTIDES IN GROWING POTATO TUBERS" PLANTA, SPRINGER VERLAG, DE, vol. 205, no. 3, July 1998 (1998-07), pages 428-437, XP000997825 ISSN: 0032-0935</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/14512

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 1-12 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-12 (partially)

The present claims are directed to a method for increasing the content of a transgene-encoded biomolecule in an organism, characterised by changing the distribution of ATP and/or ADP in cells of the organism. The expression "changing the distribution of ATP and/or ADP" is merely the characterisation of the essential feature by the desired result to be achieved without indicating the means how said change should be carried out. In addition, said expression neither indicates the extent of the change nor the exact localisation where said change should take place ("in cells").

In view of this vague and broad definition of the main feature and when taking into account that nearly each reaction in the cell involves a "change of distribution of ATP" (e.g. the use of glucose in a culture medium) a meaningful search for an accordingly characterised claim over the whole range is not possible.

As a consequence, the search had to be limited to those means which were used in the present application in order to achieve the change of ATP/ADP distribution, i.e. the use of the cloned plastidiary ATP/ADP transporter.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte	nal Application No
PCT/EP 02/14512	

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
WO 9958654	A	18-11-1999	AU	4261099 A		29-11-1999
			BR	9910408 A		09-01-2001
			CA	2328394 A1		18-11-1999
			CN	1306578 T		01-08-2001
			WO	9958654 A2		18-11-1999
			EP	1078088 A2		28-02-2001
			HU	0200074 A2		29-05-2002
			JP	2002514412 T		21-05-2002
			PL	344751 A1		19-11-2001